EXHIBIT A

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Molecular cloning and expression of a human heat shock factor, HSF1

(human transcription factor/leucine zippers/polymerase chain reaction)

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ABSTRACT **Human cells respond to heat stress by inducing the binding of a preexisting transcriptional activator (heat shock factor, HSF) to DNA. We have isolated recombinant DNA clones for a human HSF (*HSF1*) by screening cDNA libraries with a human cDNA fragment. The human *HSF1* probe was produced by the PCR with primers deduced from conserved amino acids in the *Drosophila* and yeast HSF sequences. The human HSF1 mRNA is constitutively expressed in HeLa cells under nonshock conditions and encodes a protein with four conserved leucine zipper motifs. Like its counterpart in *Drosophila*, human HSF1 produced in *Escherichia coli* in the absence of heat shock is active as a DNA binding transcription factor, suggesting that the intrinsic activity of HSF is under negative control in human cells. Surprisingly, an independently isolated human *HSF* clone, *HSF2*, is related to but significantly different from *HSF1* [Schuetz, T. J., Gallo, G. J., Sheldon, L., Tempst, P. & Kingston, R. E. (1991) *Proc. Natl. Acad. Sci. USA* 88, 6911–6915].**

Organisms respond to an increase in the ambient temperature by rapid synthesis of heat shock RNAs and proteins (1–4). The regulation of heat shock gene transcription is mediated by the transcriptional activator, heat shock factor (HSF) (5, 6), which binds to heat shock response elements (HSEs) (7–9) present upstream of all heat shock genes. Although the sequence of the HSE is highly conserved among widely separated species, there are significant differences in the properties of HSF. HSF purified from the yeast *Saccharomyces cerevisiae*, *Drosophila*, and human have distinct molecular masses when measured by SDS/PAGE—150, 110, and 83 kDa, respectively (10–12)—and the proteins do not show significant immunological cross-reaction (13). The regulation of HSF activity in yeast is also different from the regulation in *Drosophila* and vertebrate cells. In yeast, HSF is bound constitutively to heat shock promoters and undergoes heat shock-dependent phosphorylation that activates the transcriptional capacity (14–18), while in *Drosophila* and vertebrate cells it is the binding of HSF to DNA that is induced upon heat shock (19). This induction of HSF binding occurs in the absence of protein synthesis (5, 6), suggesting that preexistent HSF proteins are activated by a posttranslational mechanism.

The genes encoding HSF from the yeast *S. cerevisiae* (14, 20) and *Drosophila* (22) have been cloned. We sought to clone the human *HSF* gene as an entry to the study of mammalian HSF, whose activation temperature (40°C–45°C) is set at a higher threshold than the activation temperature for yeast and *Drosophila* HSF (37°C).*

MATERIALS AND METHODS

PCR. The PCRs were carried out according to the manufacturer's conditions (Perkin–Elmer/Cetus). Either 2 or 9 μl of a cDNA reaction mixture was used for PCR in a final vol of 50 μl, with 0.5 μl each of (0.7 μg/μl) primer I: [5'-GCCGGC(N)TT(C/T)CTGGCCAA(A/G)CT(N)TGG-3'] and primer II: [5'-CTCGAGCCA(N)AG(N)AC(C/T)TC(A/G)TT(C/T)TC-3']. The reaction was programmed for 1.5 min at 94°C, 2 min at 60°C, and 3 min at 72°C; it was repeated 27 times, with a change of the melting step to 1 min at 94°C for cycles 2–28 and the last extension step at 72°C for 6 min. Reaction products (20 μl) were analyzed by agarose gel electrophoresis and ethidium bromide staining. The cDNA synthesis reaction contained (in 50 μl) 5 μl of 10× PCR buffer, 20 μl of 10 mM dNTP (each 2.5 mM), 2.5 μl of mixed oligodeoxynucleotides [p(dN)$_6$; Pharmacia], (0.2 μg/μl), 1 μl (20 units) of placental RNase inhibitor, 1.25 μl of 50 mM $MgCl_2$, 2.5 μl of murine leukemia virus reverse transcriptase (BRL), and 5 μg (HeLa) or 2 μg (*Drosophila* embryo) of poly(A)$^+$ RNA. The reaction mixture was incubated at room temperature for 10 min and at 42°C for 45 min, terminated at 95°C for 5 min, diluted with 1 vol of H_2O, and frozen at −20°C.

Screening of Human cDNA Libraries. Approximately 10^6 plaques of a human B-cell lymphoma cDNA library (gift of L. Staudt, National Institutes of Health) and a human activated B-cell cDNA library (gift of J. Kehrl and A. Fauci, National Institutes of Health; obtained through L. Staudt) in the λgt11 and Lambda ZAP vectors, respectively, were screened. Replicate filters were hybridized with ^{32}P-labeled human *HSF* PCR fragment, a 55-mer oligonucleotide: 5'-GATGTTCTCAAGGAGCTGCTCCTGGCCACGCAGGAAGCATGGGTGCTGGAACTCC-3' and a 25-mer oligonucleotide: 5'-AAGCACAACAACATGGCCAG(C/T)TTCA-3'. Filters were prehybridized with 6× standard saline citrate (SSC)/5× Denhardt's solution/0.1% SDS for 1 hr at 65°C and hybridized with labeled DNA under the same conditions for 12–16 hr. Filters were then rinsed three times with 1× SSC/0.1% SDS at room temperature for 5 min per rinse, washed in 0.5× SSC/0.1% SDS, at 65°C for 15 min, rinsed briefly in 1× SSC, blotted dry, and exposed to x-ray film for ≈16 hr. Only plaques that gave a reaction with all three probes were considered positive. After three rounds of plaque purification, the cDNA inserts were subcloned into the vector pBluescript SK− (Stratagene) for sequence determination by the dideoxynucleotide technique. The entire sequence presented is contained within a single clone (no. 108) and has been confirmed by sequencing both strands of overlapping regions from two or more independently isolated clones. The 5' ends of five clones from the B-cell lymphoma cDNA library and two clones from the activated B-cell library are nearly coincident (within 66 nucleotides). Hence, it is likely that the 5'-terminal sequences of the *HSF1* message are represented by these clones. Alternatively, the

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Abbreviations: HSF, heat shock factor; HSE, heat shock response element; ORF, open reading frame.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M64673).

G+C-rich 5′ region could represent a common block to reverse transcription that occurred in the construction of the cDNA libraries. Sequence comparisons were performed by using the University of Wisconsin Genetics Computer Group sequence analysis program BESTFIT.

Expression of Recombinant Human HSF in *Escherichia coli*. The 489- and 529-amino acid ORFs were subcloned (after introducing a *Nde* I site by site-directed mutagenesis at the presumptive initiating codon) into the expression vector pJC20 (J.C. and C.W., unpublished data). pJC20 is a derivative of pET 3C (23). No additional amino acids were introduced into the cloned HSF protein. *E. coli* BL21(DE3) cells that carry the T7 promoter under the control of a *lac* UV5 promoter were transformed with the expression plasmid carrying human *HSF* or with the vector alone. For preparation of bacterial extracts, cells were grown in LB broth containing 0.4% glucose and ampicillin (200 μg/ml) to an OD_{600} of 0.5. Isopropyl β-D-thiogalactoside was added to a concentration of 0.4 mM and incubation was continued at 37°C for 3 hr. Cell pellets resuspended in HEMG (24) containing 0.1% Nonidet P-40 (HEMGN) and 300 mM KCl were disrupted by sonication using six 20-sec pulses at 20–25 W, with chilling in ice water between pulses. Extracts were clarified by centrifugation at 10,000 × *g* for 10 min and flash-frozen in liquid N_2. Dilutions of the extracts for experiments were made in HEMGN buffer containing 300 mM KCl and 100 μg of bovine serum albumin per ml.

Mobility Shift Assay. Dilutions of an extract of *E. coli* expressing human HSF1 were incubated with 10 fmol of ^{32}P-labeled, consensus HSE [three alternating (__GAA__) modules] and subjected to a gel shift assay on a 1% agarose gel essentially as described (19).

DNase I Footprinting. The *Drosophila hsp70* gene fragment (positions −185 to +295) was 5′-end-labeled with ^{32}P at either the upper strand or the lower strand. Labeled DNA (5 fmol) was incubated with various dilutions of an *E. coli* extract containing cloned human HSF-1, an *E. coli* extract containing cloned *Drosophila* HSF (22), or *E. coli* extract only in a total vol of 20 μl, as in gel mobility shift conditions, for 10 min at room temperature. One microliter of 100 mM $MgCl_2$ and 0.03 unit of DNase I were added, and incubation was continued for 1 min. The reactions were terminated by the addition of 2% SDS/50 mM EDTA; DNA was purified and analyzed on an 8% sequencing gel.

***In Vitro* Transcription Assays.** Extracts prepared from 0- to 12-hr *Drosophila* embryos were used for transcription assays as described (22, 24, 25). As an internal control for transcription from the template carrying two HSEs, the same template with the HSEs deleted [as well as a 30-base-pair (bp) downstream region] was mixed in the reaction. RNA originating from the template lacking HSEs is thus distinguished by a 30-nucleotide decrease in size. As a further control for RNA recovery, a defined amount of RNA synthesized from a T7 promoter upstream of the hsp70 sequences inserted into pBluescript was introduced into each transcription reaction mixture along with the stop solution. Experimental details are given in ref. 22.

Northern Blot Analysis. Total cytoplasmic RNA was prepared from nonshocked or heat shocked (30 min at 45°C) HeLa cells. Poly(A)$^+$ RNA was isolated by using a poly(A) Quik mRNA purification kit (Stratagene). Three micrograms of poly(A)$^+$ RNA was separated on a 1% agarose/formaldehyde gel, blotted onto a GeneScreen membrane (DuPont/New England Nuclear), and hybridized with a pBluescript plasmid carrying the human *HSF1* cDNA, labeled with ^{32}P by the random-priming technique (Boehringer Mannheim).

RESULTS

Isolation of cDNA Clones for Human HSF1. Examination of the amino acid sequence of *Drosophila* and yeast HSF indicated that the proteins were substantially divergent, except in the regions corresponding to the DNA binding and multimerization domains. Based on the sequence similarity in these domains, we designed degenerate primers derived from each domain for PCR amplification of a segment of the human *HSF* gene. PCR amplification generated a human cDNA fragment of ≈460 bp, similar in size to fragments amplified from total *Drosophila* cDNA and the *Drosophila HSF* cDNA clone (data not shown). The amplified human cDNA was purified, sequenced, and found to be similar (but not identical) to the corresponding region of *Drosophila HSF*. This amplified fragment, and two nondegenerate oligonucleotides contained within its sequence, were used to screen cDNA libraries for the human *HSF* gene.

The DNA and predicted amino acid sequence of a putative human *HSF* cDNA (*HSF1*) is presented in Fig. 1. The cDNA sequence presented is identical over the entire length for at least two independent clones, except for a heterogeneity in the 3′ portion of the sequence. This heterogeneity is due to a stretch of 8 nucleotides (shown in white print) that is present in one cDNA clone and absent in another clone isolated from the same library. Assuming that the first methionine found in the sequence is initiating, the cDNA variant including the 8 nucleotides carries an ORF of 529 amino acids. This sequence variant was also isolated from a different cDNA library. The other cDNA (excluding the 8 nucleotides) carries the same ORF up to the point of heterogeneity, Ser-461, after which the predicted amino acid sequence diverges until a stop codon after residue 489. The occurrence of the two sequence variants in the natural RNA population has not been determined. It should be noted that an in-frame stop codon was not found in the cDNA sequence upstream of the presumptive translational start, so that the true N terminus of the protein could lie further upstream. This possibility is diminished by the essentially equal lengths of the *HSF1* cDNA sequence and HSF1 mRNA (see below). The putative human HSF1 ORFs predict proteins unusually rich in prolines (10%) and serines (13%), most of which reside in the C-terminal half and could be targets for phosphorylation *in vivo*.

Human HSF1 Expressed in Bacterial Cells Functions as a DNA Binding Transcription Factor. We expressed the 489- and the 529-amino acid ORF in *E. coli* by using the T7 RNA polymerase-dependent expression system (22, 23). Both ORFs were found to be expressed at a comparable level in *E. coli* (Fig. 2*A*). The molecular masses of the recombinant proteins for the 489- and 529-amino acid ORFs as measured by SDS/PAGE are 60 and 70 kDa, respectively, significantly higher than the predicted mass of 52,880 and 57,273, but lower than the apparent 83-kDa mass of the purified protein. The anomalous SDS gel mobility of the cloned proteins is reminiscent of similar anomalies with yeast and *Drosophila* HSF proteins (14, 20, 22). The apparent difference in mass between the natural and recombinant human HSFs may be due to posttranslational modification of the natural protein (26).

Extracts prepared from *E. coli* expressing the human *HSF1* ORFs showed specific binding to the HSE, as indicated by a gel mobility shift assay, and by DNase I footprinting on the *Drosophila hsp70* promoter (Fig. 2 *B* and *C*). The DNase I footprints produced by the 489- and 529-amino acid ORFs are essentially identical to the footprint produced by cloned *Drosophila* HSF protein, with the exception of a distinctive hypersensitive site at the downstream border of the *Drosophila* HSF footprint, which is absent in the human HSF footprint. There is no significant difference in the relative binding affinities of the 489- and the 529-amino acid ORFs, as indicated by the equivalent footprinting activity of the two proteins. At the lowest concentration of HSF1 tested, only the proximal, high-affinity HSE is occupied (see also ref. 22).

No binding to the HSE was found in extracts prepared from *E. coli* cells transformed with the expression vector alone.

We tested the ability of the cloned human proteins to function as transcription factors *in vitro* by using a heat shock plasmid template and a cell-free transcription system derived from *Drosophila* embryos (22, 24, 25). As shown in Fig. 3*A*, addition of extracts of *E. coli* expressing either the 489- or the 529-amino acid ORF caused an ≈6-fold stimulation of transcription *in vitro*. This increase, similar to that observed with cloned *Drosophila* HSF protein, is dependent on the HSE, because no transcriptional enhancement was observed when a template with the HSEs deleted was used. The ability of the 489- and 529-amino acid ORFs to function as HSE-dependent transcription factors indicates that both ORFs encode most or all of a functional human HSF1 protein.

mRNA for Human HSF1 Is Constitutively Transcribed. We determined the expression of HSF1 mRNA in normal and heat shocked HeLa cells by Northern blot analysis (Fig. 3*B*). Consistent with the presence of an (inactive) HSF protein prior to heat shock (5, 6), the levels of human HSF1 mRNA are identical before and after heat shock, indicating that the human *HSF1* gene is constitutively transcribed under non-shock conditions. The size of human HSF1 mRNA as measured against molecular size standards is 2.0–2.2 kilobases, which is essentially identical to the length of the 2156-nucleotide cDNA sequence.

Sequence Comparison with *Drosophila* HSF Reveals Four Conserved Leucine Zippers. A previous comparison of the predicted amino acid sequences of *Drosophila* and yeast HSF revealed sequence divergence over a large portion of the proteins, except for two core conserved regions (A and B) in the N-terminal half (Fig. 1 and ref. 22). Analysis of the human HSF1 sequence shows that it is more similar to *Drosophila* HSF than to yeast HSF. In the 66-amino acid region A, which includes sequences important for DNA binding (14, 20, 22), the predicted amino acid sequence of human HSF1 (residues 16–81) is 67% identical to *Drosophila* HSF and 55% identical to yeast HSF. Residues conserved in this region of human HSF include the similarity to residues in the putative recognition helix of bacterial σ factors (22, 27, 28). In region B, human HSF (residues 164–197) is 79% identical to *Drosophila* HSF and 41% identical to yeast HSF. Region B is the

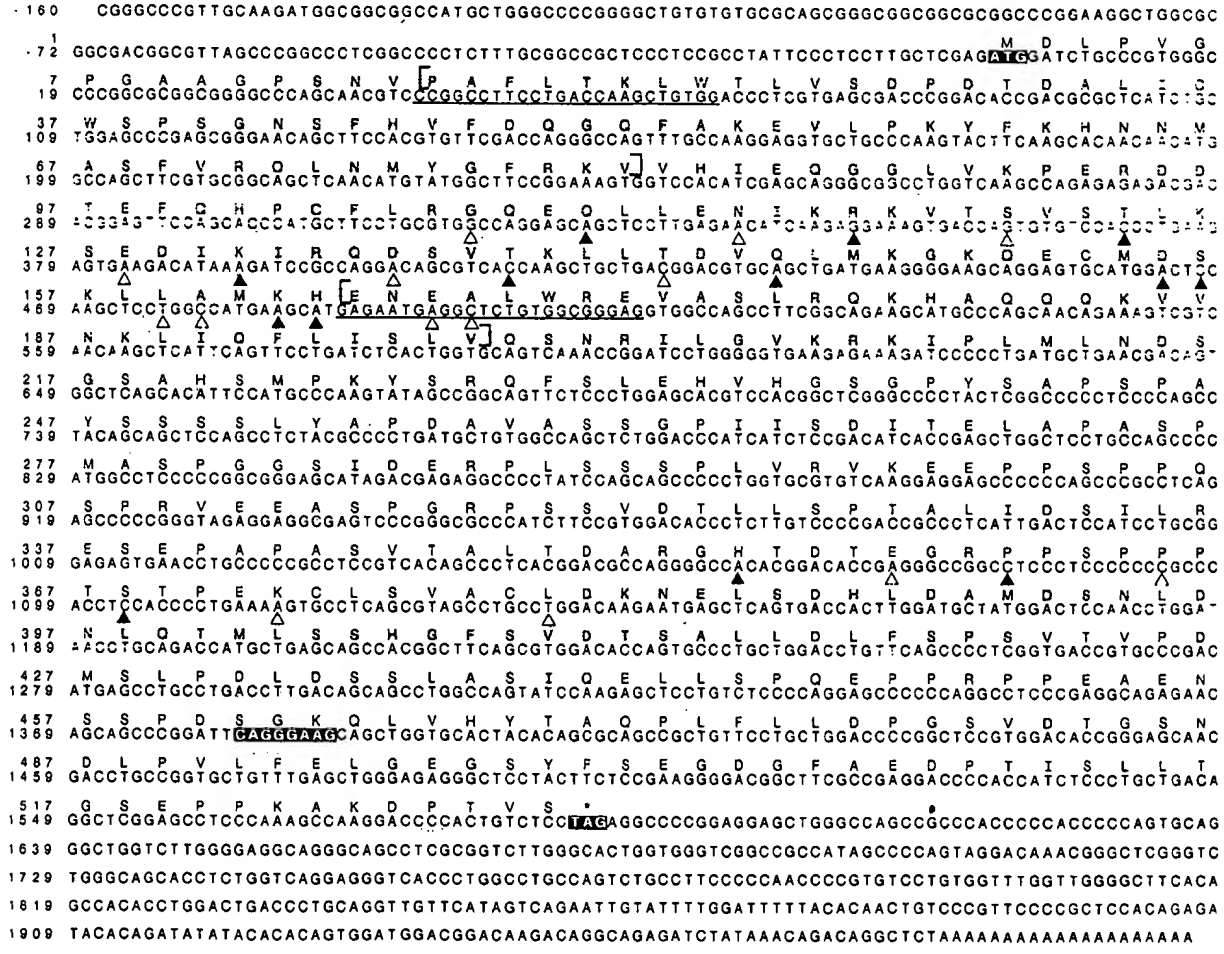

FIG. 1. DNA and predicted amino acid sequence of the human *HSF* (*HSF1*) cDNA. The presumptive start and stop codons, and the 8-nucleotide heterogeneity (see text), are highlighted in white print. Numbering of the DNA sequence shown in the left margin begins with the A of the presumptive initiating AUG codon. The amino acid sequence of the 529-amino acid open reading frame (ORF) is presented, terminated by an asterisk. The sequences corresponding to the PCR primers are underlined. Regions conserved between human *HSF1* and *HSF2* are stippled. Brackets delineate regions A (Pro-16 to Val-81) and B (Glu-164 to Val-197) corresponding to core similarities between *Drosophila* and yeast HSFs. Open and solid triangles show the four arrays of heptad repeats of hydrophobic amino acids. The 489-amino acid ORF is identical to the 529-amino acid ORF from Met-1 to Ser-461, after which the reading frame in single letter code is AGALHSAAAVPAGPRLRGHRE-QRPAGAV (amino acids 462–489). The 529-amino acid ORF variant of HSF1 shows further similarity (Lys-463 to Phe-474) to HSF2 (67% identity).

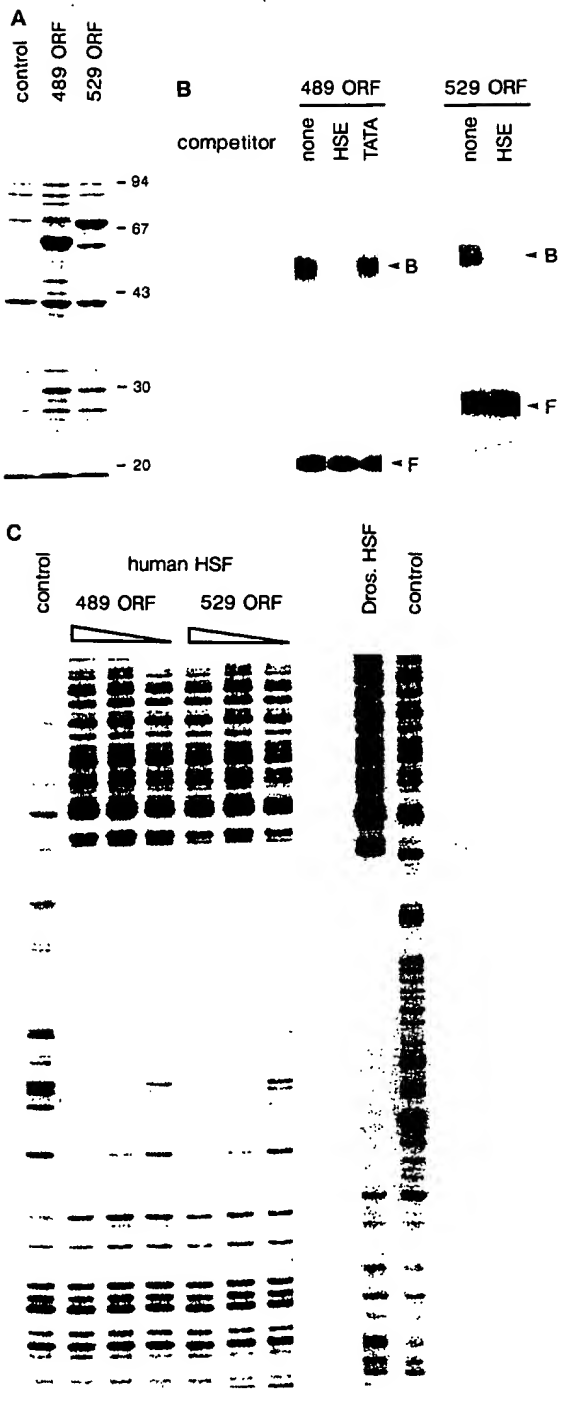

FIG. 2. Expression of HSF1 in *E. coli* and analysis of DNA binding activity. (*A*) SDS/PAGE of cloned human HSF1. Five microliters of control bacterial extracts or extracts expressing each of the two ORFs of human *HSF1* were separated on a 10% gel and visualized by Coomassie blue staining. Molecular mass standards (kDa) (Pharmacia) are indicated on the right. (*B*) Gel mobility shift analysis. One microliter of a 1000-fold (489-amino acid ORF) or 100-fold (529-amino acid ORF) dilution of bacterial extract expressing human HSF1 was incubated with labeled HSEs and analyzed by agarose gel electrophoresis. The specific activities of the labeled HSEs in the two experiments are not equivalent; hence, the -fold dilution of the bacterial extracts indicated is not an accurate reflection of the relative binding affinities of the 489- and 529-amino acid ORFs. The positions of free DNA (F) and DNA bound to HSF (B) are indicated. Assays were carried out in the absence (none) and presence of a 50-fold excess of unlabeled HSEs derived from the *hsp82* genes (19) or in the presence of sequences from the *hsp70* TATA box (TATA; ref. 11). Extracts prepared from cells not expressing HSF1 did not show HSE binding activity (data not shown). (*C*) DNase I footprinting assay. The *Drosophila hsp70* gene fragment was labeled at the 5′ end or at the 3′ end (data not shown). *E. coli* extract containing cloned human HSF1, 489- and 529-amino acid ORFs (1 μl each of undiluted, 10-fold diluted, and 100-fold diluted extract); cloned *Drosophila* HSF, Dros. HSF (1 μl of undiluted extract); or no HSF, control (1 μl of undiluted extract) was incubated with the labeled fragment and subject to DNase I digestion. Fragments were purified and analyzed on an 8% sequencing gel. Sequences protected on the upper strand are 5′-GCACACTGTTCTCGTTGCTTCGAGAGAGCGCGCCTCGAATGTTCGCGAAAAGAG-3′.

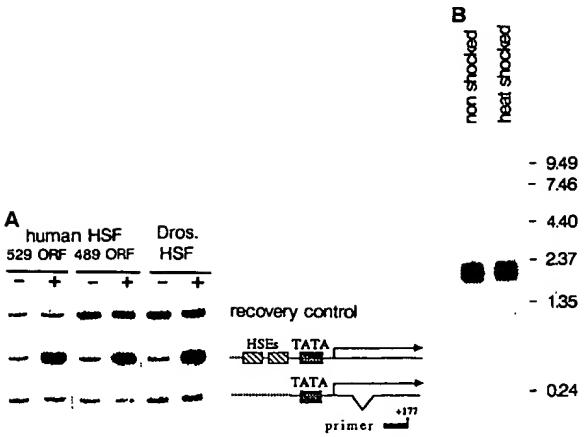

FIG. 3. *In vitro* transcription with cloned human HSF1 protein and Northern blot analysis of human *HSF1* message. (*A*) Primer-extension analysis of RNA synthesized by a cell-free transcription system derived from (nonshocked) *Drosophila* embryos supplemented with 0.1 μl of *E. coli* extract from cells expressing the 489- and 529-amino acid ORFs of human HSF (+) and from cells transformed with the expression vector only (−). For comparison, a similar experiment was performed with a 1-μl extract of *E. coli* expressing the *Drosophila* (Dros.) HSF protein. Schematic drawings of the two templates are aligned with the primer-extension products of the respective transcripts. (*B*) Expression of the human *HSF1* message. Poly(A)$^+$ RNA was fractionated by formaldehyde/agarose gel electrophoresis, transferred to a solid support, and hybridized with a labeled human HSF clone. The locations of RNA size markers (kilobases) (BRL) are indicated on the right. The amounts of mRNA from normal and heat shocked cells on the Northern blot were equivalent, as determined by probing for actin mRNA (data not shown).

conserved core of a broader region that bears three arrays of hydrophobic heptad repeats and is implicated in the multimerization of HSF. Two of these hydrophobic heptad repeats overlap and are positioned out of phase by one residue. The unusual arrangement of hydrophobic heptad repeats or leucine zipper motifs is strikingly conserved in human HSF1 and is likely to mediate the formation of multimeric human HSF1 complexes, as has been proposed for the *Drosophila* and yeast HSF proteins (22, 29). Two other regions in the C-terminal half of *Drosophila* and yeast HSF that are rich in serine and threonine residues and show marginal conservation (22) are absent in human HSF1.

We discovered a fourth leucine zipper motif located in the C-terminal region that is conserved between human HSF1 (residues 383–410) and *Drosophila* HSF (residues 583–610).

This fourth leucine zipper is absent in yeast HSF. Because the yeast HSF protein displays a constitutive DNA binding capacity, we speculate that the function of the fourth zipper in human and *Drosophila* HSFs could be to mediate formation of the inactive HSF complex under nonshock conditions. The positions of the four hydrophobic heptad repeats are denoted by triangles in Fig. 1.

DISCUSSION

We have isolated cDNA clones for a human HSF (HSF1) and shown that two variant ORFs for HSF1 function as HSE-dependent transcription factors in an *in vitro* transcription assay. These studies on the 489- and 529-amino acid ORFs were performed with protein expressed in *E. coli* at 37°C, the temperature at which HSF remains inactive in human cells. Our results suggest therefore that the cloned HSF1 proteins have an intrinsic capacity to assume the active conformation at a nonshock temperature, and this capacity is apparently repressed in human cells until the onset of heat stress. In this respect, the properties of human HSF1 parallel those of *Drosophila* HSF, which is also highly active when expressed in *E. coli* at the nonshock temperature (22). The active *Drosophila* HSF protein associates as large multimers, the hexamer being the form that binds to a canonical HSE with high affinity (22, 30), and it has been suggested that a block in multimerization, caused by altered protein folding or the binding of an inhibitory substance, may be responsible for the suppression of HSF binding *in vivo*. The cloned human HSF protein also associates as multimers (S.K.R., unpublished data); hence, a similar mode of HSF regulation might operate in human cells.

In the course of these studies, we became aware of another human cDNA cloned by Kingston and co-workers (see ref. 32). The sequence of this cDNA clone also shows similarity to *Drosophila* and yeast *HSF*, but it has substantial differences from the cDNA clones we isolated. By mutual agreement, the cDNA reported here and in the accompanying paper have been designated *HSF1* and *HSF2*, respectively. Schuetz *et al.* (32) isolated *HSF2* by screening a human cDNA library with degenerate oligonucleotides designed from the sequence of tryptic peptides of human HSF purified from heat shocked HeLa cells. Of five peptides whose sequences were determined, two are located in HSF2, whereas all five could be located without discrepancy in the common region of the 489- and 529-amino acid ORFs of *HSF1*. Therefore, it is likely that *HSF1* encodes the predominant HSF protein isolated from heat shocked human cells.

The predicted amino acid sequences of human HSF1 and HSF2 are very similar in approximately the N-terminal half of the proteins [60% identity over 206 residues, from Ser-13 to Gly-217 of HSF1, and Ser-5 to Gly-206 of HSF2 (Fig. 1)], allowing gaps of one and three residues between Asp-77 and Ser-78, and between Ser-114 and Lys-115 of HSF2, respectively, and one residue between Thr-97 and Glu-98 of HSF1. This conserved region encompasses not only regions A and B, the core similarities between yeast, *Drosophila*, and human HSFs, but also sequences extending away from the conserved cores that are divergent between HSFs of different species. By this criterion, the two human HSFs are somewhat more related to each other than to HSFs from yeast and *Drosophila*. In addition, the C-terminal region that carries the fourth leucine zipper motif is well conserved between human HSF1 and HSF2 (50% identity over 44 amino acids; HSF1 residues 379–422 and HSF2 residues 355–398) (Fig. 1). It is unlikely that the two human HSF proteins represent polymorphisms in the human population, as there are numerous nucleotide substitutions within the conserved regions and little similarity outside the conserved regions. In a parallel study, different cDNA clones were also isolated from tomato (31). Hence, the existence of multiple HSF proteins may be a property of many species.

What might be the purpose of multiple transcription factors responding to cell stress? It is possible that the different HSFs may have evolved to respond to different temperature thresholds or to other (chemical) stress signals known to induce heat shock gene transcription. Or, as observed with the two heat shock σ factors in bacteria (21, 33) and the three tomato heat shock factors (31), there may be constitutive and inducible stress regulator genes in order to accommodate transient and sustained stress signals. The availability of at least two HSF genes in humans paves the way to a detailed dissection of this important physiological response in a mammalian system.

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